GPx8

Subjects: Anatomy & Morphology
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This entry aimed to illustrate the presence of glutathione peroxidase 8 (GPx8) in rat during preimplantation period of pregnancy. Females were killed on first (D1), third (D3), and fifth (D5) day of pregnancy. The presence of GPx8 in embryos was detected under the confocal microscope, the presence of GPx8 in genital organs was confirmed immunohistochemically, and the amount of GPx8 was determined using densitometry. We found that GPx8 is dispersed in the cytoplasm of oocytes, while after fertilization, it is concentrated in granules. From 4-cell stage till blastocyst, GPx8 reaction was found in the perinuclear region. In the ovary, GPx8 was seen in granulosa-lutein cells, in plasma of blood vessels, and inside Graafian follicles. In oviduct, GPx8 was detected in the plasma and in the extracellular matrix (ECM). Moreover, epithelial cells of isthmus were positive. In uterus, GPx8 was observed in the uterine glands, in the plasma, and in ECM. On D5, the enzyme disappeared from the uterine glands and appeared in fibroblasts. Densitometry revealed that the highest amount of GPx8 was on D1 and subsequently declined. To auhotr's knowledge, this is the first entry describing GPx8 presence in the oocytes, preimplantation embryos, and female genital organs in mammals. Our results improve the understanding of antioxidant enzymes presence during pregnancy in defense against oxidative stress, which is considered to be one of the main causes of infertility.

Keywords: GPx, reproduction, antioxidant enzyme

\. Introduction

GPx7 and GPx8 are very similar, and evolutionary studies suggest that they both are derived from GPx4[1]. GPxs use glutathione (GSH) to reduce hydrogen peroxide (H2O2) and other small hydroperoxides (ROOH) to water and corresponding alcohol.

Each GPxs isoform has unique features concerning their subcellular localization, tissue distribution, substrate specificity, and their apparent biological function[2], e.g., GPx4 and GPx5 play roles in male fertility. At the same time, GPx7 and GPx8 are probably involved in correct protein folding and prevention of H2O2 leakage from endoplasmic reticulum to the cytosol[3]. Although the prevention of endoplasmic reticulum stress is considered to be a major function of GPx8, its some other unknown characteristics come to the light. Experiments on cancer HeLa cell line revealed, that GPx8 expression is induced by hypoxia[4], that silencing of GPx8 impacts membrane lipid composition[5], and that GPx8 level modulates endoplasmic reticulum Ca2+ concentration and fluxes[6]. Nevertheless, these results obtained from pathologically changed cells need to be verified on healthy cells that these mechanisms work also under physiological conditions.

2. GPx8 Expression

In the work, researchers found that enzyme GPx8 was present during the preimplantation period of pregnancy in all stages of new individuals, from unfertilized oocytes through zygotes to blastocysts. In addition, GPx8 was at the same time found in the ovary, uterine tube, and uterus of the mother. Moreover, the highest amounts of the protein in examined genital organs were on the first day of pregnancy, and then this amount declined.

Unique among the glutathione peroxidases family members, both GPx7 and GPx8 reside in the endoplasmic reticulum (ER). Unlike GPx7, which is entirely located inside the ER lumen, GPx8 is a transmembrane protein, with its active site facing the lumen[7]. The main source of ROS in the ER comes from intra-molecular disulfide bond formation during the maturation process of many secretory and membrane proteins. This process requires protein disulfide isomerase (PDI) and endoplasmic reticulum disulfide oxidase 1α (Ero1α) with subsequent H2O2 production[8][9]. However, such H2O2 cannot diffuse from ER to cytosol owing to the peroxidase activity of GPx8. This mechanism is essential to protect cells from Ero1α-mediated hyperoxidation and death[10]. Since embryos, during their development, produce large amounts of proteins needed for their growth and differentiation, it was not surprising that they contain also the GPx8.

According to our findings, GPx8 was observed in its typical localization around the nuclei, where the ER is situated, from the 4-cell stage to the blastocysts. Nevertheless, the protein was also detected in ovulated oocytes and zygotes up to the 2-cell stage. In these earlier stages of new individual development, GPx8 was dispersed in the cytoplasm. Thus, differences between maternal and embryonic GPx8 genome expression are evident. Briefly, oocytes accumulate a large set of proteins derived from the maternal genome. These maternal proteins are not only required for oocyte maturation and fertilization, but later, most of them are degraded, and their amino acid components are utilized for the synthesis of new proteins based on the embryonic genome[11]. In rodents, the start of embryonic gene transcription, called the zygotic genome activation (ZGA), accelerates in the transition from 2-cell to 4-cell stage, as opposed to the transition from 4-cell to 8-cell stage in humans[12]. Knowing differences in GPx8 distribution patterns, its enzymatic activity during maternal genome expression could be questioned since the enzyme was homogenously diffused in the cytoplasm. After fertilization, the enzyme even began to be concentrated in granules distant apart, and such protein arrangement resembles some kind of building framework. Indeed, this protein arrangement exists in GPx4, a vitally needed antioxidant enzyme, which is considered to be a direct ancestor of GPx8. GPx4, in mid-piece of mature spermatozoa, is a chemically inactive form and acts as a structural protein[13]. However, one can speculate that in related enzymes in a specific condition and specific cell types, similar arrangement could also be possible.

Of all known GPxs, only GPx3 and GPx5 have been described as extracellular enzymes so far. GPx3 is synthesized mainly by the kidney and released into the blood, where it is proposed to be a major scavenger of ROS because it accounts for nearly all of the glutathione peroxidase activity in plasma[14]. GPx3 from the plasma can also traverse the blood vessels and bind to basement membranes in many tissues[15]. GPx5 is released from epithelial cells into the epididymal lumen to protect maturing mammalian spermatozoa from OS[16]. Because GPx8 is anchored in the ER membrane, the presence of this enzyme in the blood plasma and also in perivascular connective tissue was surprising. The true role of this protein here is unclear, but one can hypothesize that GPx8 could be involved in the maintenance of protein disulfide isomerase (PDI), which in ER acts in protein folding, since GPx8 and GPx7 may accept PDI as a reductant more efficiently than does GSH. Moreover, a recently published study suggested that PDI is present in human plasma at important levels and could be even used as a marker for some medical conditions[17].

On the other hand, ROS, and especially H2O2, are important regulators of endothelial cell homeostasis, which modulate their proliferation, migration, survival, and vasorelaxation[18]. Blood vessels are surrounded by extracellular matrix (ECM), which is an important microenvironmental component that modifies the kinetics of H2O2 consumption; therefore, it might be important during angiogenesis, endothelial cell migration and endothelial cell survival, and homeostasis regulation. This supports the existence of a crosstalk between ECM-dependent signaling and redox signaling to direct endothelial cell behavior[19]. ECM is a complex supramolecular material that includes collagens, elastin, proteoglycans, and glycosaminoglycans restricted to the basement membrane and interstitial spaces of all tissues[20]. ECM plays not only a role as a building network, but it is considered as an active structure in cell migration, division, and differentiation[21]. The ECM is degraded by matrix metalloproteinases (MMPs), which play important roles in tissue remodeling of female genital organs during cyclic changes, such as ovulation, menstruation, pregnancy, or cervical dilation during labor. Tissue inhibitors of metalloproteinases (TIMPs) act contrary to MMPs. ROS could trigger the tissue MMP activation and subsequent ECM degradation in the process of human trophoblast invasion[22] or rupture of fetal membranes, a major cause of preterm birth[23]. Previous studies have also indicated that MMP3, MMP-9, and TIMP-1 might participate in oviduct remodeling during the menstrual cycle[24], whereas MMP-8 activity participates in tissue remodeling processes during inflammation to establish successful Gonorrhea infection[25]. From this perspective, it is not surprising that we found GPx8, a member of the antioxidant enzyme family that degrades H2O2, in the ECM of the rat oviduct and uterus. Female genitals regularly undergo tissue remodeling during menstruation or pregnancy, so the balance between MMPs and TIMPs must also be maintained with the presence of antioxidant enzymes. Such equilibrium is essential for tissue stability because extensive and destructive degradation of the ECM could be seen in various pathological conditions, such as arthritis or cancer[26]. The only problem is that the GPx8 enzyme has not been described so far to be freely located in the ECM. We can assume that the real significance of the GPx8 presence in the plasma is not the H2O2 degradation or involvement in PDI maintenance in the bloodstream but the passage through the vessel walls into the ECM of the target organs. This is because we were not able to detect the presence of the enzyme in any cells immediately adjacent to the GPx8 detected in the ECM. Nevertheless, to prove or decline our working hypothesis, other types of experiments are needed.

The corpus luteum contains high levels of antioxidant enzymes, including SODs and GPxs, which protect luteal cells against ROS produced during steroidogenesis[27]. On the other hand, these oxygen radicals may also be functional in leading to luteolysis and apoptosis in corpus luteum during each reproductive cycle after prostaglandin F2 Alpha (PGF2α) stimulation[28]. Even exogenous hydrogen peroxide has been shown to inhibit progesterone synthesis in rat granulosa-lutein cells[29]. Since ROS are known to be involved in luteolysis[30], the corpus luteum requires antioxidant protection. It is

known that when granulosa cells differentiate into corpus luteum, there is accompanying hypertrophy of the agranular endoplasmic reticulum, which seems to be further enhanced during pregnancy. This hypertrophy of the endoplasmic reticulum appears to reflect an increased demand for steroidogenesis and is also responsible for increased total protein observed in the pregnant corpus luteum[31][32]. Our finding that GPx8 is also present in granulosa lutein cells probably suggests that increased steroidogenesis in the corpus luteum needs protection against OS development.

From previous research, it is clear that some levels of H2O2 in the ovary are essential for ovulation to occur and for correct oocyte development[33]. The surge of LH, which is responsible for ovulation, also stimulates elevated ovarian ROS production[34]. On the other hand, the reduced reproductive outcome was recorded in oocytes retrieved from a follicular fluid (FF) exposed to higher H2O2[35]. However, the limit between signaling and harmful H2O2 level is very small. It was estimated that 60 ng of ROS/oocyte is enough to maintain oocyte in diplotene arrest, whereas just a moderately increased ROS production of 80 ng causes meiosis resumption in oocytes[34]. Hence, it is not surprising that we detected GPx8 in Graafian follicles inside the ovary by immunohistochemistry and in ovulated oocytes and corona radiata cells by immunofluorescence in the oviduct. Probably, as the H2O2 production increases during follicular growth, demand for the presence of antioxidant enzymes involved in fine-tuning of the redox balance increases. Similarly, GPx1 protein was identified in bovine granulosa cells of large but not small healthy follicles, and the GPx1 gene expression was significantly higher in human cumulus cells from cumulus-oocyte complexes yielding a pregnancy[36].

The isthmus of the oviduct in mammals acts as a sperm reservoir, which is created by the binding of uncapacitated spermatozoa to the epithelial lining[37]. Such interactions increase the activities of antioxidant enzymes in spermatozoa, and sperm can survive here for up to several days[38]. Moreover, transcripts that encode GPxs enzymes were present in the mouse and human oviducts[39], but regional differences were observed. In cows, GPx3[40] and GPx4[41] were under-expressed in the ampulla and over-expressed in the isthmus. This is because the ampulla is the site of fertilization, and spermatozoa need ROS presence for the capacitation to occur, while spermatozoa in the isthmus need to be protected against ROS-induced damage as they constitute a sperm reservoir[42]. High GPxs activity was also detected in cow oviductal fluid. However, which GPxs would be expressed in the uterine fluid was unclear[40]. In our work, we detected GPx8 in the secretory cells of the isthmus. There are two possible reasons for the enzyme presence. First, that enzyme is released into the oviductal lumen, and second, the enzyme remains in the cells as the protection against OS, since secretory cells have intensive metabolism, and they are responsible for the secretion of many proteins and other factors which contribute to the formation of the oviductal fluid[43]. A similar situation was observed in cells of uterine glands that synthesize and secrete many substances, such as enzymes, growth factors, hormones, and transport proteins, collectively termed histotroph, into the uterine cavity, which subsequently influences blastocyst implantation and conceptus survival in mammals [44]. At this stage, the real role of the enzyme in different cells of the female genital system is unclear. However, at least in the secretory cells of the oviduct and in the cells of the uterine glands, the enzyme could be secreted into the lumen since we detected it as granules in the apical parts of the cells. On the other hand, in fibroblasts of endometrium and fimbriae of the Fallopian tube, the enzyme probably remains in the cells as the protection against OS since it was observed in the whole cytoplasm. In the rat uterus, the stimulus provided by the presence of embryo triggers the process of decidualization, when endometrial stromal fibroblasts proliferate and differentiate into decidual cells[45]. The decidua plays an essential role in protecting the embryo from being attacked by maternal immune cells and provides nutritional support for the developing embryo before placenta formation. The decidua also secretes many hormones, growth factors, and cytokines, such as prolactin, relaxin, or GnRH[46].

During the ovulation, the infundibulum covers the site of follicular rupture, and the fimbriae catch and conduct the oocytes into the oviductal lumen. One of the specific function of fimbriae is their chemotactic activity to estradiol levels from mature follicular fluid[47]. A limited number of studies have focused on the fibroblasts in the fimbriae; hence, it is not clear why GPx8 positive fibroblasts are situated in this part of the oviduct. Since fibroblasts synthesize and secrete the precursors of all the components of the ECM[48], the different components of the ECM in fimbriae, compared to other parts of the oviduct, might potentially influence their different motility and mucosal condition[49].

Concerning WB, we found that GPx8 is present in all observed organs. The main band of the protein was detected at approximately 57 kDa, and in salpinx and uterus, additional two bands of higher size were detected. This could indirectly prove that the enzyme is also located in sperm or components of seminal plasma, as these additional two bands have gradually disappeared with advancing pregnancy and were never observed in the ovary. Unfortunately, neither a sperm nor seminal plasma have not been analyzed in direct corroboration with our hypothesis. On the other hand, the actual band sizes for the GPx8 differ from predicted (24 kDa), probably due post-translational modification, such as phosphorylation or glycosylation, which both can increase the size of the proteins. The possibility, that protein forms

dimers or trimers cannot also be excluded, as it was already proposed in hamster kidney with GPx[50]. Nevertheless, the nature of the protein and the molecular weight detected by Western blot will require a further demonstration of GPx8 by mass spectrometry.

Densitometry revealed that the highest amount of the enzyme was present in all organs on D1 of pregnancy when also ovulation occurs. On D3, in the amount of the protein decreased, and this decline continued in the ovary and uterus on D5 as well. On the other hand, in the salpinx, a successive mild increase of GPX8 amount was recorded on D5. Possible explanations for this phenomenon are that in the further course of pregnancy, higher ROS levels are necessary, or that other isoforms of GPXs or even CAT could replace GPx8 function.

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